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Positional Cloning of Genes Involved in the Beckwith-Wiedemann Syndrome, Hemihypertrophy, and Associated Childhood Tumors

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The Beckwith-Wiedemann syndrome (BWS) is an overgrowth malformation syndrome that occurs with an incidence of 1:13,700 births. There is a striking incidence of childhood tumors found in BWS patients. Various lines of investigation have localized "imprinted" genes involved in BWS and associated childhood tumors to 11p15. High resolution mapping of 8 rare balanced chromosomal BWS rearrangements enabled us to identify three distinct regions on chromosome 11p15 that might harbor genes involved in the above-mentioned disorders. These results suggest genetic heterogeneity that correlates with the clinical heterogeneity seen in the patients studied. Expressed candidate gene sequences from these regions have been cloned and partly sequenced. These transcripts are either disrupted by or are at least within a few kb of these BWS chromosome breakpoints. So far, zinc-finger sequences and one Kruppel-associated box (KRAB) domain were found in independent candidate genes which are compatible with a regulating function of growth promoting genes. The abundance of expression of these genes varies from low abundant in all adult and fetal tissues tested to detectable on Northern blots of adult tissues. In addition to our 11p15 studies we have analyzed additional chromosome regions, in particular 1p. Cytogenetic, loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) studies have identified 1p35 as a region of interest. A positional cloning effort to identify a balanced 1p35 translocation found in a Wilms tumor has led to the isolation of a YAC, crossing this breakpoint.

Key words: childhood tumors, Beckwith-Wiedemann syndrome, hemihypertrophy, positional cloning, chromosome 11p, chromosome 1p

INTRODUCTION

The Beckwith-Wiedemann syndrome (BWS) was first described independently by Beckwith (1963) [1] and Wiedemann (1964) [2]. The syndrome occurs with an incidence of 1:13,700 births and is characterized by numerous growth abnormalities, especially the examphalos (umbilical hernia), macroglossia (enlarged tongue) and gigantism triad, explaining the EMG acronym for this syndrome. These features are variably present and can be found in association with multiple abnormalities including neonatal hypoglycemia (low blood glucose levels), typical ear creases and pits, and a unilateral growth abnormality of parts of the body called hemihypertrophy.

There is a striking increase of at least 7.5% in the incidence of different types of tumors found in BWS patients, including the following childhood tumors: Wilms' tumor (59% of all tumors found in this disorder), adrenocortical carcinoma (15%), and a few instances of hepatoblastoma and rhabdomyosarcoma [3].

BWS has been assigned to chromosome region 11p15 by linkage analysis, near the insulin (INS) and insulin-like growth factor 2 (IGF2) genes. Overproduction of these genes has been noted in some BWS cases. In addition, loss of constitutional heterozygosity (LOH) limited to chromosome region 11p15 was found in BWS-associated tumors. Both the syndrome and the associated tumors are subject to genomic imprinting [4 and references therein].

Positional Cloning of Balanced BWS-Associated Chromosomal Breakpoints

Our cytogenetic analysis of 8 balanced chromosomal rearrangements found in BWS patients revealed break-

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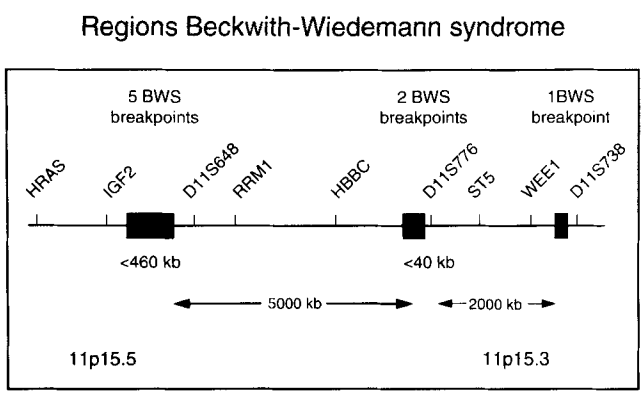

Fig. 1. Beckwith-Wiedemann chromosome regions (BWSCR). Five breakpoints (BWSCR1) are mapped to 11p15.5 within a 460-kb DNA fragment just proximal to IGF2 and distal to RRM1. Two breakpoints (BWSCR2) are located 5 Mb more proximal within a DNA fragment of at most 40 kb, close to D11S776. One breakpoint (BWSCR3) is mapped an additional 2 Mb more proximal between the markers D11S572 and D11S738.

points in the distal region of 11p15. Most of them seemed to cluster in 11p15.5; a few breakpoints were mapped more proximal at 11p15.3. In order to investigate these breakpoints in more detail at the molecular level, a physical map of this region was constructed. Using a combination of pulsed field gradient electrophoresis (PFGE) and fluorescent in situ hybridization (FISH) techniques, we were able to construct a contiguous map of 10 Mb. Several hundred markers were positioned on to this map and their position relative to the BWS breakpoints was established [4–6]. In this way we could position the breakpoints on the map (Fig. 1).

Five breakpoints clustered at 11p15.5 within a 460-kb fragment near, but clearly distinct from, the imprinted genes *IGF2* and *H19*. We preliminarily designated this region BWSCR1 (Beckwith-Wiedemann syndrome chromosome region 1). All patients presented with the classical BWS symptoms. Two additional breakpoints were mapped 5 Mb more proximal of BWSCR1. These breakpoints (BWSCR2) were associated with patients demonstrating hemihypertrophy, in one case Wilms' tumor, but both had only minor signs of BWS. One additional breakpoint was mapped 2 Mb proximal of BWSCR2. This breakpoint (BWSCR3) was associated with a patient showing all classical symptoms of BWS. These findings suggest that multiple genes are involved in the development of the syndrome, whereas genetic heterogeneity might correlate with clinical heterogeneity (BWSCR1/3 versus BWSCR2).

Figure 2 summarizes our cloning results in BWSCR1. All breakpoints, including a rhabdoid tumor breakpoint mapped to this region, were contained within a DNA fragment covered by three overlapping yeast artificial chromosomes (YACS). Subcloning of these YACS into cosmids enabled us to construct a contiguous set of overlapping cosmids throughout this region. Cosmids overlapping the individual breakpoints were identified by FISH and Southern blot analysis. Evolutionary conserved CpG islands (CG-rich sequences, often involved in gene regulation) from these cosmids were cloned and used as probes on Northern blots. These probes recognize a major 6.5-kb transcript in various adult tissues (especially skeletal muscle and heart) and less abundantly in fetal tissues (Fig. 2B). Screening of cDNA libraries resulted in the isolation of 5 partial cDNAs in this region so far. Sequence analysis of these clones and various genomic clones from this region is in progress. Homology to a known expressed sequence tag (EST) was found. In addition, hybridization of the BWSCR1 cosmids to an oligonucleotide specific for the linker region of zinc-binding finger motifs (ZnF) revealed a cosmid positive for the oligonucleotide. This, however, has to be confirmed by sequence analysis.

Figure 3 summarizes our cloning results in BWSCR2. Again, we subcloned a YAC overlapping both breakpoints into cosmids. A single cosmid overlapping both breakpoints could be identified. Genomic sequence analysis revealed an open reading frame between both breakpoints and zinc-binding finger motifs flanking the breakpoints. The transcription of these zinc-finger motifs, however, is in opposite directions and they are therefore coding for different genes. Expression of these sequences is only detectable with reverse transcriptase polymerase chain reaction (RT-PCR) techniques and not with Northern blot analysis. Sequence analysis of RT-PCR products of the gene with four zinc-finger motifs furthermore revealed a KRAB (Krüppel-associated box) domain 5′ of the zinc-finger motifs. This motif has been associated with repression of transcription in transfection experiments [7,8]. Finally, a poly adenylation tail was found close to, but not actually crossing the breakpoint associated with BWS, hemihypertrophy and Wilms' tumor. At present, this latter sequence is thus a strong candidate gene but its involvement in the above-mentioned disorders has to be proven. The same holds for other transcribed sequences near these BWSCR2 breakpoints.

With PFGE techniques we were able to place the BWS breakpoint within BWSCR3 to a 990-kb AscI fragment. FISH analysis with cosmids from this region enabled us to place the breakpoint between two evolutionary conserved sequences, one of which was also positive after hybridization to the oligonucleotide specific for the linker region of zinc-binding finger motifs. This latter finding has to be confirmed with sequence analysis.

Chromosome 1P and Childhood Tumors

The association of BWS and Wilms' tumor points to the occurrence of tumor suppressor genes on chromosome 11p15. Cytogenetic changes found in Wilms' tumors, however, suggest the involvement of other chromosomal regions, such as 1p, 16q and 17p, in the etiology of this tumor [9]. We have focused our studies on chromosome 1p since, in our hands, 7 out of 30 (23%) Wilms' tumors

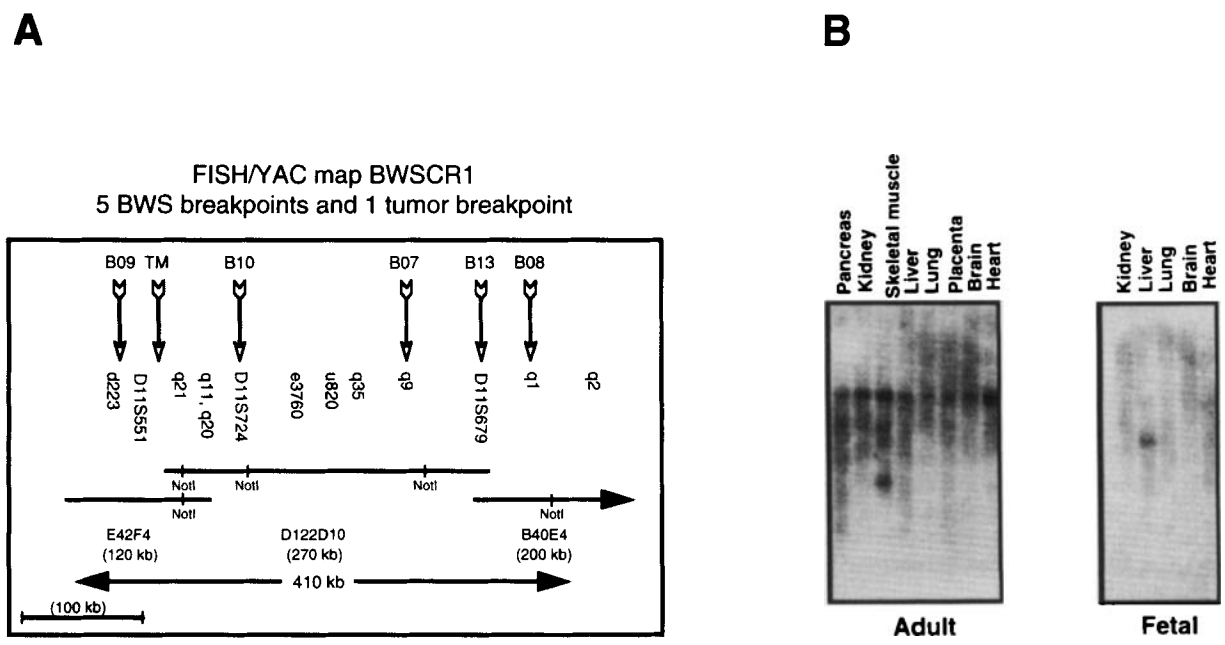

Fig. 2. **A:** Schematic representation of BWSCR1. The DNA markers used are shown. The five BWS breakpoints and one rhabdoid breakpoint (TM87-16) are indicated with arrows and the cosmids overlapping the breakpoints are listed above. NotI sites within YACs used are shown. Cosmids q1 and q9 were subcloned; CpG islands from these cosmids were evolutionary conserved and used as probes on Northern blots. **B:** Northern blot analysis of single copy probes from q1 (shown), q9 and 3 partial cDNAs (not shown). All probes recognize a major 6.5-kb transcript in various adult tissues (especially skeletal muscle and heart) and less abundantly in fetal tissues.

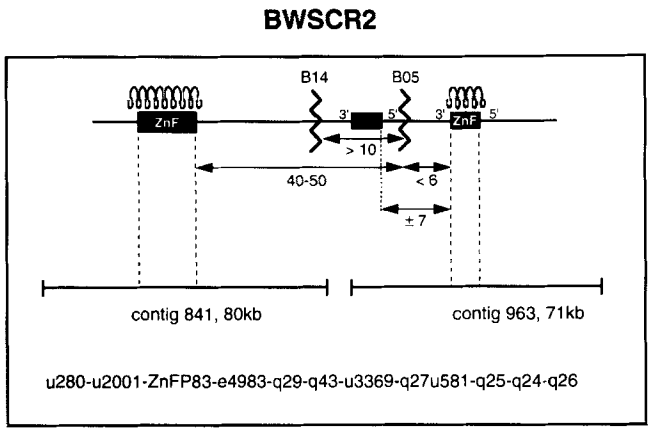

Fig. 3. Schematic representation of the location of the hemihypertrophy and BWS-associated breakpoints (B05 and B14) within BWSCR2. B05 is also associated with the development of Wilms' tumor. Open reading frames (ORFs) are shown in black boxes. The zinc-finger motifs are shown. Estimated distances and the order of cosmids within the cosmid contigs is given.

tested showed 1p allele losses. With LOH and CGH studies we were able to demonstrate genetic alterations on 16q, 7p and some other chromosomes, however, to a minor extent (<13%).

Figure 4 shows a summary of our studies on 1p. Two distinct regions were found to be involved in allele losses that might represent two regions involved in tumor development (comparable with the situation found in neuroblastoma). However, given the small number of cases analyzed, allele loss in the proximal region might also be due to complex genetic rearrangements. Of interest is that the most proximal was found to be deleted in a liver metastasis of a Wilms' tumor and not in the primary tumor (WT59M in Fig. 4). In addition, 1p LOH was also found in premalignant tissue (nephrogenic rests) of Wilms' tumors.

Within the more distal region, we were able to map balanced chromosomal rearrangements found in a Wilms' tumor, congenital mesoblastic nephroma and rhabdomyosarcoma. For our cloning efforts we focused on the Wilms' tumor breakpoint since this was the only cytogenetic abnormality found in this tumor. We collected 78 random cosmids within region 1p35 and mapped 25 of these relative to the breakpoint with FISH. This enabled us to place the breakpoint between D1S56/ALPL/FUCA and the proximal marker D1S60. We then were able to use an existing YAC contig for FISH analysis and we could place the breakpoint within the overlap of 2 YACS.

Conclusions

Positional cloning has led to the identification of a number of 11p and 1p breakpoints involved in BWS, hemihypertrophy and/or associated childhood tumors. Transcribed candidate sequences within two distinct

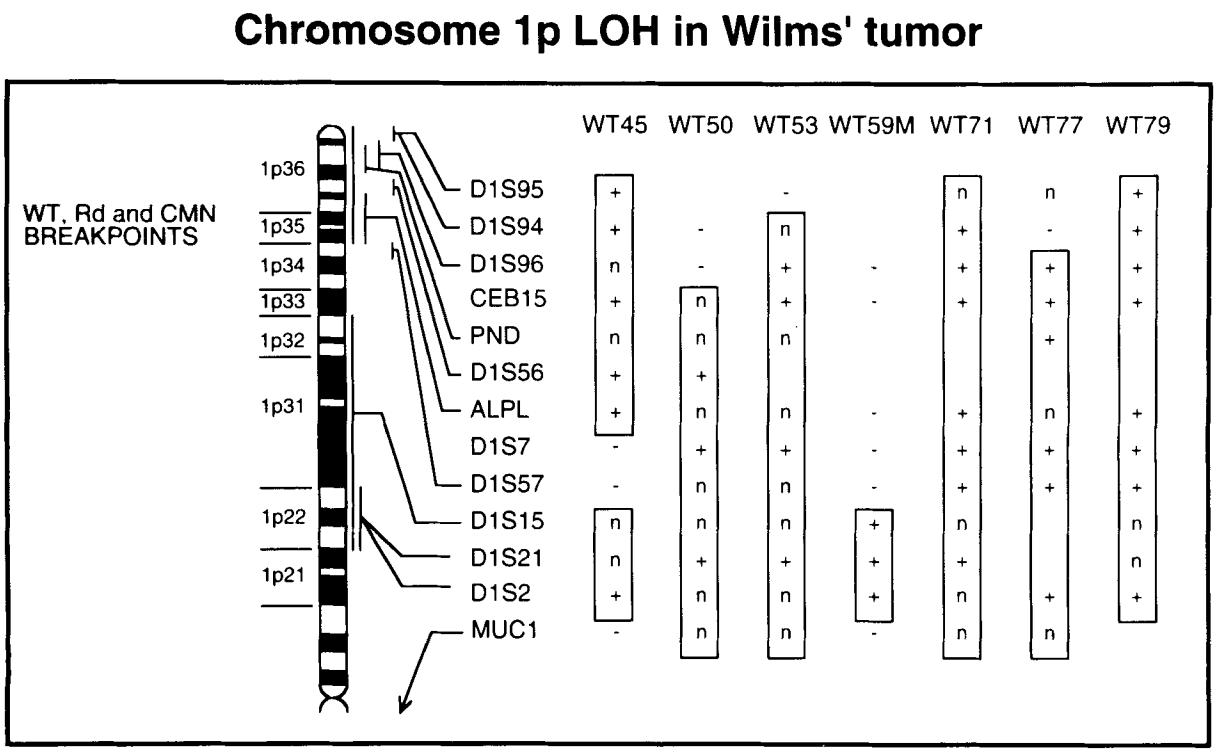

Fig. 4. + = Loss of heterozygosity (LOH); − = retention of heterozygosity; n = not informative. Thirteen chromosome 1 markers were used to map the smallest region of overlap (SRO) of LOH in Wilms' tumor. In four tumors, the most telomeric markers are not deleted. Tumor WT45 shows LOH for two distinct regions on 1p, a distal region (1p36-1p35) and a more proximal region (1p32-centromere). The liver metastasis (WT59M) only shows LOH for markers of the more proximal region (deletion 1p32-p31 confirmed with CGH). The translocation breakpoints in a Wilms' tumor (WT), a rhabdomyosarcoma (Rd) and a congenital mesoblastic nephroma (CMN) within 1p35 are indicated.

breakpoint clusters on 11p have been cloned that either overlap, or are within close vicinity of these breakpoints. Preliminary data suggest that these candidate sequences contain motifs that are compatible with a regulating function of growth-promoting genes.

Our results suggest genetic heterogeneity that correlates with the clinical heterogeneity seen in the patients studied ("classical" BWS phenotype versus mild BWS and hemihypertrophy phenotype).

Cytogenetic, LOH and CGH studies suggest the existence of genes on 1p, involved in BWS associated childhood tumors, in at least two distinct regions.

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Commentary

Mannens et al. suggest that multiple genes are involved in the development of Beckwith-Wiedemann syndrome (BWS), explaining the clinical heterogeneity of this disease. Analyzing the breakpoints of 8 balanced chromosomal rearrangements found in as many patients with BWS. They have identified three distinct regions on chromosome 11p15 that might contain genes involved in BWS and childhood tumors, including Wilms tumor, that are associated with this overgrowth disorder. Five patients with breakpoints in a region designated BWSCR1 (Fig. 1) presented with classical BWS symptoms; both patients with breakpoints in BWSCR2 (proximal to BWSCR1) demonstrated only minor signs of BWS, while one patient with a breakpoint in BWSCR3 (proximal to BWSCR2) again showed all the classical symptoms of BWS. Finally, these investigators also report on their efforts to identify genes involved in the development of Wilms tumor and other BWS associated tumors, on chromosome 1p.